

Short Communication

MOLECULAR INVESTIGATION OF LUMPY SKIN DISEASE VIRUS IN CATTLE AROUND INDORE DISTRICT OF MADHYA PRADESH, INDIA

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ABSTRACT: A study was conducted to know the etiology of cutaneous lesions induced by infectious agent of a disease which were found prevalent in and around Indore district of Madhya Pradesh, India, during 2020-21. Affected cattle showed febrile illness with the presence of nodules all over the body, malaise, pain at site of lesion and enlarged superficial lymph nodes. Skin samples were collected from unorganized and organized farms, gaushalas and slaughterhouse located at Mhow cantonmentboard, after detailed clinical examination. A total of thirty suspected cases were reported. Infected cattle had lesions on neck, back, perineum, tail, limbs, genital organs and mucous membranes. Skin samples were collected from the infected animals. Laboratory investigations included detection of lumpy skin disease virus by polymerase chain reaction (PCR), virus isolation and electron microscopy (EM). Phylogenetic analysis based on the nucleotide sequences of ORF103 gene revealed that India-MP strain sequence showed 100% homology and it was observed that the sequences aligned with the Kenya strain of lumpy skin disease virus. The virus was isolated from six clinical specimens and lumpy skin disease virus particles could be observed in electronmicroscopy. The isolate had been found was LSDV-MP/India/21 ORF103.

Keywords: Lumpy skin disease virus, Scab, PCR, Phylogenetic analysis, Electron microscopy.

Large number of diseases have been reported to pose a threat to public health, veterinary professionals and other peoples coming in contact with domestic animals like cattle and buffaloes, one of such emerging vector born viral disease is Lumpy skin disease. This disease has been reported to cause huge morbidity and mortality among cattle all over the world. The disease is caused by Lumpy skin disease virus (LSDV) of genus capripoxvirus of subfamily chordopoxvirinae and family poxviridae. The capripoxvirus genus comprised of Lumpy Skin Disease virus, sheep pox virus and goat pox virus. The main mode of transmission of this virus is through the bite of blood feeding arthropod vectors [1]. The genome of this virus could be detected by RT-PCR in subclinically infected cattle using ear notch and

skin biopsies [2]. The major symptoms of this infectious viral disease affecting cattle are fever, nasal and lacrimal discharge, ulcers in the eye, swollen lymph nodes and a drop in milk production. In first published report of LSD in India it was found that out of 2539 animals, 182 were positive with no mortality but having 7.1% morbidity and the isolate responsible for the outbreak in Odisha was caused by a KSGPO-like vaccine strain [3]. The disease has been confirmed in Ratlam, Ujjain, Mandsaur, Neemuch, Betul, Indore and Khandwa districts in the year 2021. The symptoms of this disease have also been reported in animals in Dhar, Burhanpur and Jhabua districts.

Thus, awareness of the skin diseases with reference to farming practices will provide opportunities for future

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disease control to enhance animal welfare and rural prosperity. Keeping this in view, the present study was undertaken to investigate the molecular characteristics and presence of Lumpy Skin Disease virus in cattle populations around Indore district of Madhya Pradesh, India.

The study

Sampling area

Cattle showing cutaneous lesions were screened irrespective of sex, age and breed from unorganized and organized farms and gaushalas including around 10 villages in and around Indore district of Madhya Pradesh, India namely Pedami, Devguradiya, Simrol, Borkhedi, Sutarkhedi, Mhow, Gopalpura, Kakriya, Manpur. Pithampur and slaughterhouse located at Mhow cantonment board. Clinical details were recorded from affected animals and skin biopsies were collected during field visits. Laboratory investigations were carried out at pox virus lab., Division of virology, Indian Council of Agriculture Research–Indian Veterinary Research Institute, Mukteshwar (Uttarakhand) and at Sophisticated Analytical Instrumentation Facility (SAIF), All India Institute of Medical Sciences (AIIMS), New Delhi.

Sample collection

A total of 12 skin biopsies were collected aseptically from clinically diseased cattle showing well-circumscribed raised cutaneous nodules on their body. Samples were collected and preserved in buffered glycerol solution, stored at -20°C and also in 2.5% glutaraldehyde solution, stored at 4°C for virological investigation.

Processing of tissue samples

DNA extraction

The skin scabs were processed as 10% (w/v) homogenates for viral DNA extraction using DNA Sure® Tissue Mini Kit – Genetex Biotech Asia Pvt. Ltd. according to manufacturer's instructions and stored at -20°C for further use.

Amplification of ORF 103 gene for detection of LSDV

PCR was carried out to amplify fragment of ORF103 gene of LSD virus using specific primers. Primers for the specific amplification of the ORF103 gene sequence were designed using the Oligo 6.0 software based on a published SPPV (10700-99 strain) genomic sequence available in the GenBank (accession No. AY077832)

and is presented in Table 1. The PCR reaction was set up by adding 2.5 μl of 10X PCR buffer, 2.0 μl of MgCl_2 (10 mM), 1.0 μl of forward and reverse primer (10pmol/ μl) each, 1.0 μl of dNTPs mix (10mM each), 2.0 μl of DNA template, 0.5 μl Taq DNA polymerase (5units/ μl) and the reaction mixture was made up to 25 μl using 15 μl nuclease free water (Table 2). The reaction mixture was placed in thermocycler (BioRad, California) with initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 30 sec, annealing at 52°C for 40 sec, extension at 72°C for 45 sec and final extension at 72°C for 10 min and hold at 5°C for 4 min (Table 3).

Visualization of PCR product

The amplified PCR products were further electrophoresed in an agarose gel (in TBE buffer), stained with ethidium-bromide solution. For electrophoresis, 1% agarose gel prepared in 1X TBE buffer containing 0.5 $\mu\text{l}/\text{ml}$ of ethidium bromide. Before loading the gel the casting platform was placed on leveled platform and open sides were sealed with the adhesive tape. The gel comb was then placed across the platform so that the teeth of comb remained 1mm above the base of the platform. The molten agarose was poured and kept undisturbed to solidify the gel. When gel was solidified, comb was removed. Gel submerged in electrophoresis tank with the wells at the cathode end of the tank with sufficient quantity (about 1mm level) of buffer (1X TBE) above the surface of the gel. The electrophoresis was carried out at 80 volts/cm for 45 minutes along with the test sample, known positive amplified product and gene ruler DNATM ladder plus 100 bp (MBI, Fermentas). The resolution of bands in the gel was visualized by a UV transilluminator and digitally recorded by gel documentation system.

Sequence analysis

The PCR product was got sequenced commercially from Eurofins Genomics Pvt. Ltd. The sequences were analyzed using BLAST analysis. Further the sequences were compared with other sequences available in GenBank, National Centre for Biotechnology Information (NCBI) and phylogenetic analysis was done using Mega 7.0 software.

Virus isolation

The skin scab was processed to make 10% suspension in PBS along with antibiotics followed by freezing thawing, filtration and storage at -80°C for further use. Vero cells grown in eagle's minimum

essential medium (EMEM) supplemented with antibiotics and 10% fetal calf serum were infected with filtered scab suspension using the adsorption method. The cells were observed daily for 6 days for the appearance of cytopathic effect (CPE) shown by LSD virus which is confirmed by PCR as described above.

Transmission electron microscopic (TEM) study

Skin biopsies of LSD affected animals were aseptically collected in sterile tubes in 2.5% glutaraldehyde solution and stored at 4 °C and sent to AIIMS, New Delhi to establish the presence of LSD virus by ultra-sectioning of tissues. Samples were transferred to vials and fixed in 2.5% glutaraldehyde solution in 0.05M phosphate buffer at PH 7.2. Tissue pieces of 1-2 mm in size were immersed in this solution for 24 hours at 4-10 °C. For post fixation of samples were placed in 1% aqueous osmium tetroxide for 2 hours at 4-10 °C. After post fixation, the tissues were dehydrated by immersing it sequentially in a series of graded ethyl alcohol such as 30, 50, 70, 90 percent and absolute alcohol. The dehydration fluid was replaced by clearing fluid such as propylene oxide (two changes of 15 min each) and tissues were embedded in hard spurr's resin block. Both semi thin and ultra thin sections were cut with a glass knife on ultra microtome. Semi thin sections of 200-300 nm thickness were stained with toluidine blue and ultra thin sections 50-70 nm thickness were mounted on grids. Then the sections were stained with saturated aqueous Uranyl acetate and counter stained with 4% lead citrate and introduced into the specimen chamber of the Transmission Electron Microscope column for observation.

Results and discussion

A total of 6 scabs from 30 suspected cases were collected. Then genomic DNA were extracted from the samples using DNA extraction kit and subjected to ORF103 gene specific PCR. All samples were found positive for LSD by PCR using ORF 103 gene specific primers, yielding of a product size of 592 bp when visualized in gel documentation system. The PCR amplification of ORF103 gene of LSD virus is presented in Fig. 1. Phylogenetic analysis revealed that

all LSDV sequences from both the locations are making a monophyletic tree with already reported LSDV from India. For the sequence analysis, one PCR product of India MP strain (partial ORF103 gene) was sequenced, these were analyzed using NCBI BLAST and it was observed that LSDV India-MP strain sequence (sample) showed 100% homology with ORF103 gene of Lumpy Skin Disease virus. Sequence was submitted in GenBank NCBI (Accession no. OR050593). When the sample sequence were compared with other sequences from various part of the world available in GenBank using clustal W in MEGA 7.0 software, it was observed that the sequences aligned with the Kenya strain of LSDV. The phylogenetic analysis of one tissue sample further proved that the sample sequence (India, MP strain) and other isolates from different parts of country formed a similar clad with Kenya strain sequences of LSD virus and the isolate found was LSDV-MP/India/21 ORF103. PCR positive samples were then inoculated into Vero cell line by pre-adsorption method of infection for virus isolation. LSD virus exhibited cytopathic effect *viz.*, rounding and clumping of cells, increased refractility, degeneration and detachment of cells after two passages (Fig. 3). Transmission Electron Microscopy of LSD affected skin tissues revealed LSD virus developing virions in the cytoplasm of a large histiocytic cell, including immature spherical forms and brick like mature forms (UA & LC x 2895) as shown in Fig. 4.

The present study confirmed the presence of LSD virus infection in cattle around Indore district of M.P. through conventional PCR which correlates with the findings of different workers *viz.*, Mafirakureva *et al.* [5] who studied molecular characterization of LSD virus using P32 gene. The P32 gene was amplified by using simple PCR and amplification products consistent with the expected amplicon size (192 bp) were obtained; Sevik *et al.* [6] and Sudhakar *et al.* [3] also performed real time PCR in Turkey and Odisha, respectively. Lakshmi Kavitha *et al.* [7] studied clinico-molecular diagnosis and characterization of bovine LSD virus in Andhra Pradesh. Phylogenetic analysis based on nucleotide sequencing of LSD virus (LSDV) ORF011, ORF012 and ORF036 suggested that the isolated virus

Table 1. Primers for amplification of ORF103 gene of LSD virus [4].

Primer type	Sequence	Position in genome
Forward primer	5'- ATGTCTGATAAAAAATTATCTCG -3'	99085-99107
Reverse primer	5'- ATCCATACCATCGTCGATAG -3'	98538-98557

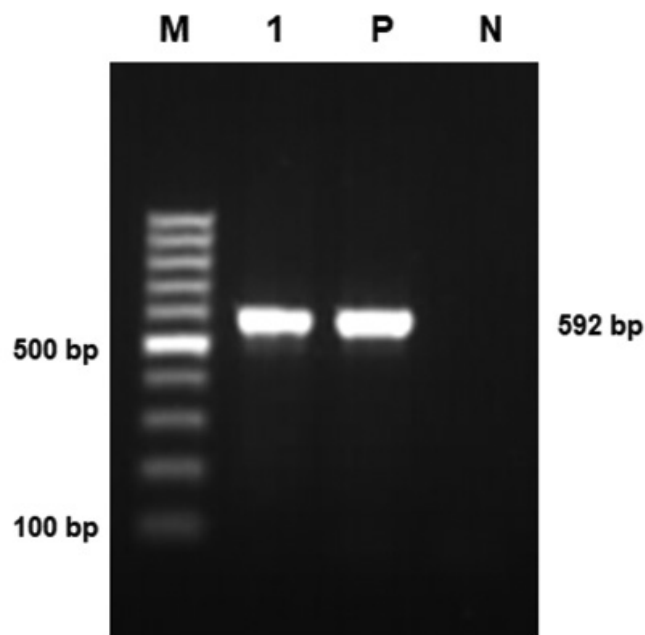


Fig. 1. PCR amplification of ORF103 gene of LSD virus.
[Lane M: 100 bp DNA ladder (Fermentas); Lane 1: LSDV-MP;
Lane P – Positive control; Lane N: No template control].

(LSDV/*Bos taurus*-tc/India/2019/Ranchi) is closely related to Kenyan LSDV strains [8]. Additionally further confirmation of LSD virus infection were done through cell culture and TEM as described by various worker's viz., Fay *et al.* [9] who described the replication and spread of LSD virus on Madin-Darby bovine kidney (MDBK) cells and the formation of foci-type poxvirus plaques on MDBK cells and also described the methods utilizing MDBK cells to quantify neutralizing antibodies and to purify LSD virus genomic DNA suitable for short read sequencing. Further, Kumar *et al.* [8] first describes the successful isolation of LSD virus in India, who adapted the isolated virus in Vero cells. Infection of the isolated LSD virus to Vero cells did not produce cytopathic effect (CPE) until the 4th blind passage, but upon adaptation, it produced high viral titres in the cultured cells. The kinetics of viral DNA synthesis and one-step growth curve analysis suggested that Vero cell-adapted LSD virus initiates synthesizing its genome at ~24 hours post-infection (hpi) with a peak level at ~96 hpi whereas evidence of progeny virus particles was observed at 36–48 hours (h) with a peak titre at

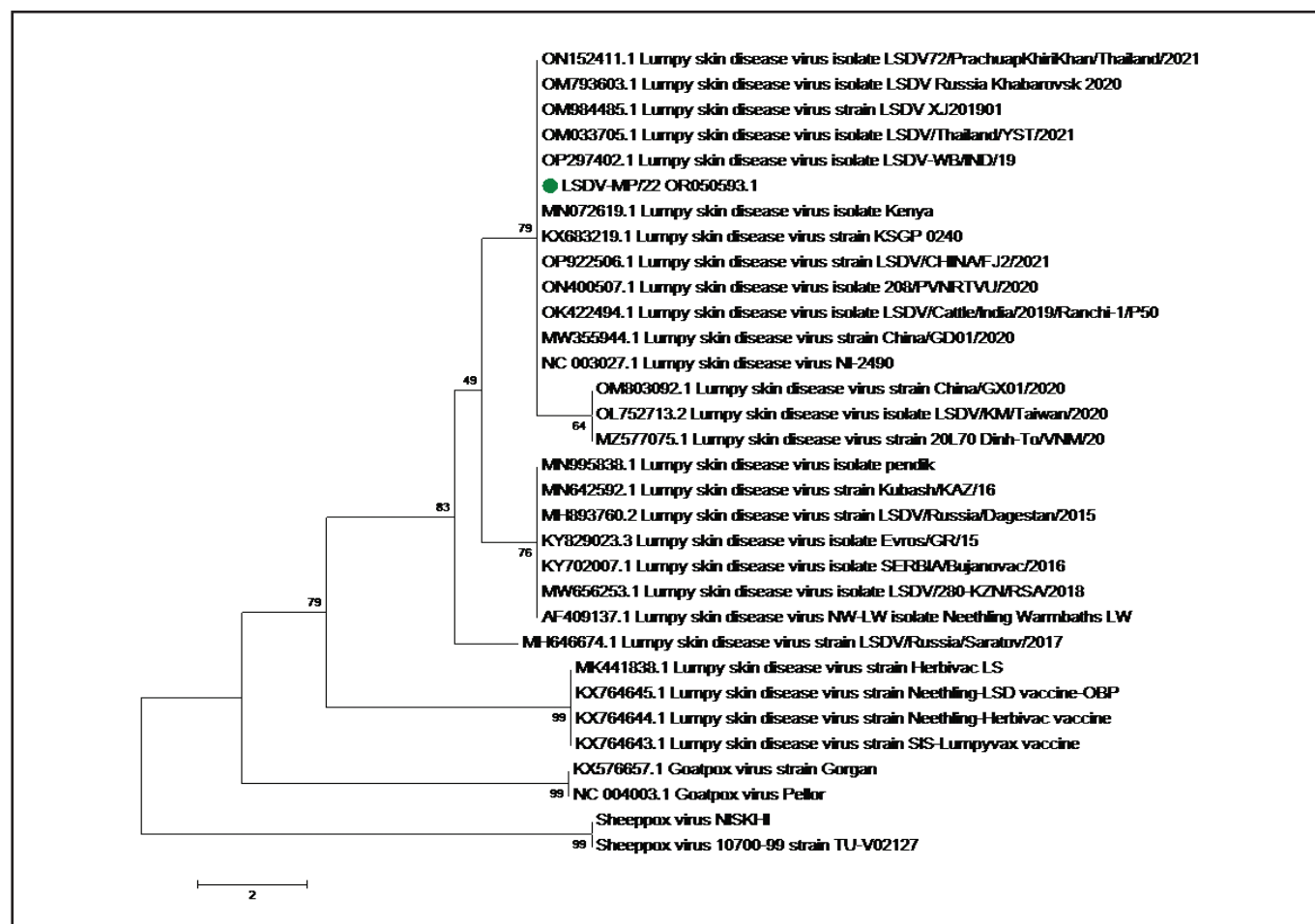


Fig. 2 . Phylogenetic analysis based on ORF103 gene.

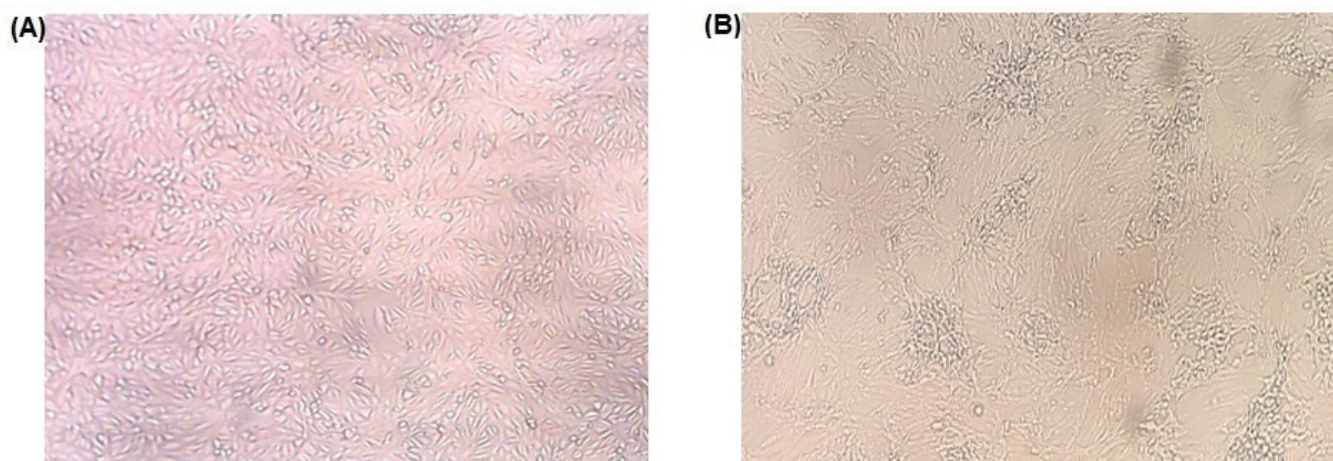


Fig. 3. Panel A: Healthy Vero cells; Panel B: LSD virus showing cytopathic effect at 5 dpi, [rounding and clumping of cells, increased refractility and degeneration].

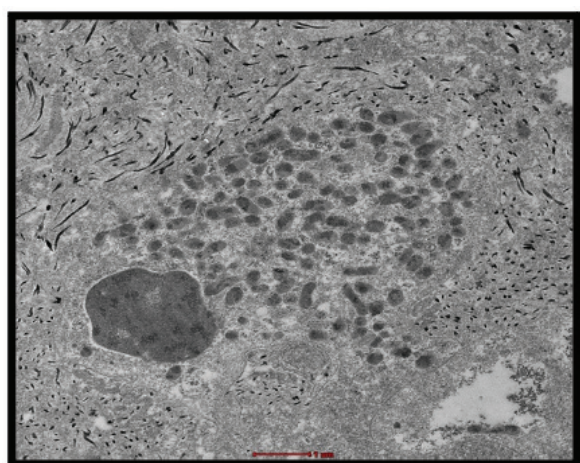


Fig. 4. TEM of skin showing intracellular immature virions (black arrow) and mature virions (white arrow) in a large histiocytic cell. [UA & LC x 2895].

~120 h. Following experimental infection of LSD virus intracytoplasmic inclusion bodies were identified in the infected histiocytes in the skin nodules of affected cattle by TEM [10]. Poxvirus-like particles were observed within the homogenate of skin nodules of diseased cattle by negative stain electron microscopy [11].

Conclusion

The PCR approach proved specific for the detection of LSDV and confirmative diagnosis of the disease. The PCR product of India MP strain (full length ORF103 gene) was got sequenced and it was observed that LSDV India-MP strain sequence (sample) had 100% homology with ORF103 gene of other Indian Lumpy Skin Disease Virus strain. On isolation virus exhibited cytopathic effect and transmission electron microscopic study showed cytoplasmic inclusions of capripox virions.

Ethical statement: The study was approved by the Institutional Animal Ethics Committee (Reference no. 01/Ethical/veterinary/2021 Date: 21/01/2021).

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